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Assessing short-term responses of prokaryotic communities in bulk and rhizosphere soils to tall fescue endophyte infection

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Abstract In contrast to endophyte-free (E–) tall fescue, endophyte-infected (E+) tall fescue pastures appear to enhance soil carbon sequestration. A hypothetical mechanism that may account for the enhanced carbon sequestration is that the E+ tall fescue affects the soil microbial community or components of it that are involved in organic carbon turnover. A 60-week mesocosm study with a factorial arrangement of soil type, loamy sand (LS) and clay loam (CL), and E+ and E– tall fescue was conducted to determine if the soil microbial communities were affected by the presence of the endophyte. Bulk and rhizosphere soil samples were fixed in paraformaldehyde, and prepared for total direct microbial counts, and with a combination of one of a domain or subdivision fluorescent oligonucleotide probe for enumerating metabolically active Eubacteria, bacterial subdivisions, and Archaea. E+ tall fescue suppressed the archaeal and high G+C gram-positive bacterial communities of the bulk CL, the delta-proteobacterial community in the rhizosphere CL, and the Planctomycetes community of the rhizosphere LS. In the long-term, suppression of these microbial communities may be a factor in enhanced soil carbon sequestration associated with E+ tall fescue.

Keywords Archaea · Carbon sequestration · Endophyte · Eubacteria · Fluorescence in situ hybridization · Tall fescue

Abbreviations
E+ endophyte-infected
E– endophyte-free
LS loamy sand
CL clay loam

Introduction

Tall fescue (*Lolium arundinaceum* Schreb. S.J. Darbyshire) is an important grass grown for forage and turf around the world. It has a natural association with an endophytic fungus, *Neotyphodium coenophialum*, which grows in the above ground parts of the plant and produces alkaloids toxic to cattle, sheep, and horses. Because tall fescue infected with this fungal endophyte is more resistant to overgrazing and disease than uninfected tall fescue, the fungal endophyte is considered an important component in tall fescue's agroecological fitness (Clay 1997).

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Franzluebbers et al. (1999) reported that total C and N contents of soil under endophyte-infected tall fescue (80% seed infection) were greater than soil under low endophyte-infected (<7% seed infection) tall fescue. They also observed that potential C mineralization per unit of total C was lower under high endophyte-infected than low endophyte-infected tall fescue pastures. Based on these observations we hypothesized that a connection existed among the observed increase in soil carbon sequestration associated with endophyte-infected tall fescue, reduced C mineralization, and composition of the soil microbial community.

In this paper we tested the hypothesis that the density of metabolically active bacterial communities differed in soils of endophyte-infected tall fescue compared to endophyte-free tall fescue. Our experimental approach was to determine if the components of the soil microbial community affected by the presence of the endophyte could be discerned and enumerated with domain specific (Eubacteria and Archaea) and eubacterial subdivision specific (Alpha-, Beta, Gamma, and Delta-Proteobacteria, high G + C gram-positive bacteria, Cytophaga-Flavobacteria, and Planctomycetes) fluorescent oligonucleotide probes developed and tested for in situ whole cell hybridization (Amann 1995; Amann et al. 1995). We used the domain oligonucleotide probes for the eubacterial and archaeal soil communities because they targeted most subdivisions of bacteria and Archaea, respectively. We wanted to obtain an overall estimation of their cell densities compared to total microbial counts, and to determine if E+ tall fescue affected the overall metabolic activities of the microbial community. We chose the nucleotide probes for the various bacterial subdivisions to investigate in more detail if E+ tall fescue affected any of the particular bacterial subdivisions since they are known components of the soil microbial community (Zarda et al. 1997), and play functional roles in various soil processes, chemical transformations, and mineralization of inputs from primary production.

A mesocosm experiment was conducted by planting E+ and E– tall fescue into two soil types of differing particle size distribution. The two soil types were chosen to test for a soil texture effect on the distribution of the components of the microbial community targeted by the oligonucleotide probes.

Materials and methods

Experimental setup

An outdoor mesocosm study consisting of 48 experimental units was conducted from March 2002 until April 2003 near Watkinsville GA (33°52′ N, 83°25′ W). The experimental design consisted of a randomized factorial arrangement of soil type (clay loam [CL] and loamy sand [LS]) and E+ and E– tall fescue to make four treatments E+ CL, E+ LS, E– CL, and E– LS in which three replicates of each treatment were sequentially harvested at 8, 20, 36, and 60 weeks of growth.

Experimental details were described in Franzluebbers (2006). Soil was collected locally from a field dominated by Cecil-Pacolet-Appling series (clayey, kaolinitic, thermic Typic Kanhapludults) at an excavated site representing subsoil at ~1 m depth (CL) with 32% clay, 22% silt, and 46% sand, and at a drainage way representing alluvial wash (LS) with 24% clay, 13% silt, and 63% sand.

Tillers of 2–3-year-old 'Jesup' tall fescue plants were excavated the morning of the start of the experiment (5 March, 2002) from two adjacent pastures containing E– and E+ tall fescue and washed free of soil. Ergot alkaloid concentration of forage at the end of the experiment was below detection limit under E– tall fescue for both soils, and was 16.7 ± 0.8 μg g^{-1} under E+ tall fescue with no difference between soils, and, thus, verified the absence and presence, respectively, of the endophyte. Five tillers were placed in each experimental unit with soil in 176 cm^3 containers. Initial soil bulk density was 1.1 and 1.2 Mg m^{-3} for CL and LS, respectively. Containers were irrigated twice per week with 0.4 l pot^{-1} with either a nutrient solution containing 7.5 mg NH_4–N, 7.5 mg NO_3–N, 35 mg urea-N, 50 mg P_2O_5, 50 mg K_2O, 0.5 mg chelated Fe, 0.25 mg chelated Mn, 0.25 mg chelated Zn, and 1.25 mg humic acid l^{-1} or with tap water when needed to avoid desiccation. From December 2002 to February

2003, containers were moved into an unheated greenhouse to avoid complete dormancy during the winter. At 8, 20, and 36 weeks of growth, those experimental units not removed for plant and soil analyses had the forage clipped ~3 cm above the soil and placed on the soil surface to decompose.

Soil sampling, fixation, dispersal in preparation for total counts and hybridization

At each of the four sampling dates, 12 experimental units were removed for analysis (three replicates of each treatment). Soil was mixed and separated from roots. All soil loosened from the roots was considered bulk soil, and the soil adhering to the roots after gentle shaking was considered rhizosphere soil. To remove rhizosphere soil from the roots of the tall fescue plants, roots were washed with 1 l of ultrapure water to produce a soil slurry. Aliquots of soil suspension were dried for one day at 105 °C to obtain dry weights of the soils sampled for analysis. Preparation of bulk and rhizosphere soil samples followed the method of Zarda et al. (1997).

Total microbial counts

The protocol for preparing filters for microscopic observation and total direct microbial counts was that described by Zarda et al. (1997) with some modifications. After filter preparation, it was transferred to a glass slide (a drop of immersion oil was placed on the slide and the filter on top of the oil) and mounted under a 22-mm^2 cover glass in 7.5–10 µl of a glycerol and Prolong Antifade (Molecular Probes, Eugene, OR) and examined with a Leica DMR fluorescent microscope equipped with filters to observe 4′-6-diamidino-2-phenylindole (DAPI) stained cells and a 100 × /1.40–0.7 oil PL APO DIC (differential interference contrast) objective and 10 × eye pieces. Twenty-five fields (out of >10^5 possible fields) were randomly selected and counted. Total count g soil^{-1} and detection threshold were determined as average counts × dilution factor × area of filter/area of microscopic field (0.01 mm^2).

Whole-cell hybridization

After Zarda et al. (1997), 10 µl of the fixed, dispersed soil sample was spotted on a gelatin-coated slide (0.1% gelatin, 0.01% $KCr(SO_4)_2$) and allowed to dry at room temperature for 6–8 h followed by dehydration in 50, 80, and 96% EtOH for 3 min each. To each spot of sample on the slide, 10:l of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS (pH 7.2), and 10–35% formamide depending on the probe) (Table 1), and 2 µl of probe (25 ng µl^{-1}) and 5 µl DAPI (200 ng ml^{-1}) were added, and then incubated in a high humidity chamber at 48 °C for 2–3 h, followed by rinsing three times with ultrapure water and air-dried. Slides were then mounted with 10 µl of Prolong Antifade (Molecular Probes, Eugene, OR) covered with a 22-mm^{-2} cover glass, and examined with a Leica DMR fluorescent microscope as described above.

Table 1 Oligonucleotide probes, their target microorganisms, their nucleotide sequences, and ratio of % formamide (FA) to mM NaCl

Probe	Target	Sequence	%FA mM NaCl^{-1}	Reference
Eub338	Eubacteria	5'-CGTGCCTCCCGTAGGAGT	30/102	Amann et al. (1990b)
Arch915	Archaea	5'-GTGCTCCCCCGCCAATTCCT	20/308	Stahl and Amann (1991)
Alf1b	α-Proteobacteria	5'-CGTTCGYTCTGAGCCAG	10/440	Manz et al. (1992)
Bet42a	β-Proteobacteria	5'-GCCTTCCCACTTCGTTT	30/102	Manz et al. (1992)
Gam42a	γ-Proteobacteria	5'-GCCTTCCCACATCGTTT	30/102	Manz et al. (1992)
SRB385	(-Proteobacteria	5'-CGGCGTCGCTGCGTCAGG	20/308	Amann et al. (1990a)
HGC69a	High G + C gram+	5'-TATAGTTACCACCGCCGT	20/308	Roller et al. (1994)
CF319a	*Cytophaga-Flavobacteria*	5'-TGGTCCGTGTCTCAGTAC	35/80	Manz et al. (1996)
Pla5a	Planctomycetes	5'-GACTTGCATGCCTAATCC	30/102	Zarda et al. (1997)

Table 2 Pooled mean total cell counts (TC), and pooled mean counts of Eubacteria (Eub), Archaea (Arch), Alpha- (Alf), Beta- (Beta), Gamma- (Gam), and Delta- (SRB) Proteobacteria, high G+C gram-positive (HGC), Cytophaga-Flavobacteria (CF), and Planctomycetes (Pla) in bulk soil for the four treatments (1) E+ LS, (2) E– LS, (3) E+ CL, and (4) E– CL after tall fescue was planted

Main Effect/Contrast	Treatment	TC	Eub	Arch	Alf	Beta	Gam	SRB	HGC	CF	Pla
		Log_{10} cells g $soil^{-1}$									
	E+ LS	8.9^{a}	8.4^{b}	8.0^{b}	7.8^{b}	7.5^{a}	8.3^{b}	7.7^{a}	$7.3^{a,b}$	7.0^{a}	7.4^{a}
	E– LS	8.9^{a}	8.5^{b}	8.0^{b}	7.9^{b}	7.9^{a}	$7.7^{a,b}$	7.6^{a}	$7.4^{a,b}$	7.0^{a}	8.0^{a}
	E+ CL	8.9^{a}	7.9^{a}	7.1^{a}	$7.4^{a,b}$	7.4^{a}	7.5^{a}	7.3^{a}	7.1^{a}	7.1^{a}	7.7^{a}
	E– CL	8.8^{a}	$8.2^{a,b}$	7.8^{b}	7.1^{a}	7.0^{a}	7.6^{a}	7.7^{a}	7.6^{b}	7.1^{a}	7.3^{a}
		Pr > F									
SW		<0.0001	<0.0001	0.15	0.38	0.36	0.001	0.29	0.004	0.75	0.59
Treatment		0.51	0.08	0.008	0.02	0.24	0.18	0.48	0.10	0.87	0.27
SW × Treatment		0.18	0.20	0.50	0.87	0.43	0.83	0.71	0.28	0.75	0.90
CL versus LS		0.59	0.02	0.007	0.004	0.18	0.13	0.39	0.95	0.46	0.49
E+ versus E		0.57	0.27	0.09	0.54	0.36	0.52	0.51	0.04	0.81	0.82
Interactions		0.19	0.52	0.11	0.36	0.20	0.13	0.27	0.16	0.81	0.07

Results of analysis of variance for main effects and interactions (sampling week [SW], Treatment, and SW by Treatment), and contrasts (CL verus LS, E+ verus E–, and Interactions between soil type and endophyte infection) for categories of cells are presented

Pooled means followed by different letters indicates a significant difference at $P < 0.10$

Data analysis

With the exception of the total count data, nearly all of the data from the FISH analyses displayed a Poisson distribution. Data were transformed for statistical analysis using the equation $X' = (X + 0.5)^{1/2}$ (Zar 1999). Means of the transformed data were back transformed, converted to numbers of cells g $soil^{-1}$, and these mean cell densities were transformed into natural log numbers before performing statistical analysis and comparing means for significant differences (at $P < 0.10$) with Proc Mixed of SAS (version 8.2). After analysis data were transformed into log_{10}.

Results and discussion

Total cell counts

Before tall fescue was planted, mean total cell count (TC) was 8.8 log_{10} cells g^{-1} of LS, and 8.7 log_{10} cells g^{-1} of CL. Endophyte and soil type had no effect on bulk soil TC (Table 2), or on the rhizosphere soil TC (Table 3). As expected, rhizosphere soil TC was greater by an order of magnitude than bulk soil TC ($P < 0.001$). The greater cell counts for the rhizosphere soil treatments were expected as part of a general rhizosphere effect (Alexander 1977). Sampling week affected both bulk and rhizosphere soil TC (Tables 2 and 3). Increases in bulk soil TC were observed between weeks 20 and 36 (Fig. 1A) and can be attributed to root growth and deposition of C with time (Franzluebbers 2006). Differences in rhizosphere soil TC between sampling weeks on the other hand appeared to be random (Fig. 1B).

Eubacterial cell counts with Probe Eub338

In situ hybridization with the domain specific oligonucleotide probe Eub338 demonstrated within the two soil types that E+ tall fescue did not have a significant effect on eubacterial cell counts in bulk (Table 2) and rhizosphere (Table 3) soils. Significant differences were observed between sampling weeks for both bulk (Table 2) and rhizosphere (Table 3) soil eubacterial cell counts. Increases ($P \leq 0.05$) in bulk soil eubacterial cell counts occurred between sampling weeks 8 and 20, and then decreases ($P \leq 0.05$) occurred in cell counts between sampling weeks 20 and 36 (Fig. 2A); whereas, increases ($P \leq 0.05$) in cell counts for the rhizosphere soil eubacteria occurred between sampling weeks 20 and 36 (Fig. 2B). Eubacterial cell counts in bulk LS were greater than the eubacterial cell

Table 3 Pooled mean total cell counts (TC), and pooled mean counts of Eubacteria (Eub), Archaea (Arch), Alpha- (Alf), Beta- (Beta), Gamma- (Gam), and Delta- (SRB) Proteobacteria, high G+C gram-positive (HGC), Cytophaga-Flavobacteria (CF), and Planctomycetes (Pla) in rhizosphere for the four treatments (1) E+ LS, (2) E– LS, (3) E+ CL, and (4) E– CL

Main Effects/Contrast	Treatment	TC	Eub	Arch	Alf	Beta	Gam	SRB	HGC	CF	Pla
		Log_{10} cells g $soil^{-1}$									
	E+ LS	9.7 ^{a}	9.8 ^{b}	9.1 ^{b}	8.5 ^{b}	8.5 ^{b}	8.3 ^{b}	9.0 ^{a}	9.0 ^{b}	9.3 ^{c}	9.6 ^{b}
	E– LS	9.5 ^{a}	9.6 ^{a,b}	9.2 ^{b}	8.3 ^{b}	8.2 ^{a,b}	8.5 ^{b}	9.1^{a}	9.2 ^{b}	8.9 ^{b}	9.5 ^{b}
	E+ CL	9.7 ^{a}	9.5 ^{a,b}	8.4 ^{a}	7.9 ^{a}	8.0 ^{a}	8.1^{a,b}	8.8 ^{a}	8.5 ^{a}	8.1^{a}	9.2 ^{a,b}
	E– CL	9.6 ^{a}	9.2 ^{a}	8.6 ^{a}	8.1^{a}	8.1^{a}	7.9 ^{a}	8.7 ^{a}	8.5 ^{a}	8.1^{a}	8.7 ^{a}
		Pr > F									
SW		0.05	.0001	<0.0001	<0.0001	0.01	<0.0001	0.08	0.07	0.0003	0.0003
Treatment		0.51	0.11	0.002	0.06	0.14	0.10	0.70	0.02	<0.0001	0.22
SW by Treatment		0.58	0.86	0.14	0.27	0.01	0.004	0.30	0.04	0.03	0.43
CL versus LS		0.61	0.07	0.0002	0.02	0.05	0.02	0.27	0.003	<0.0001	0.08
E+ versus E–		0.18	0.09	0.29	0.92	0.43	0.91	0.85	0.72	0.33	0.34
Interactions		0.63	0.05	0.76	0.23	0.28	0.28	0.75	0.47	0.13	0.52

Results of analysis of variance for main effects and interactions (sampling week [SW], Treatment, and SW by Treatment), and contrasts (CL versus SL, E+ versus E–, and Interactions between soil type and endophyte infection) for categories of cells are presented

Pooled means followed by different letters indicates a significant difference at $P < 0.10$

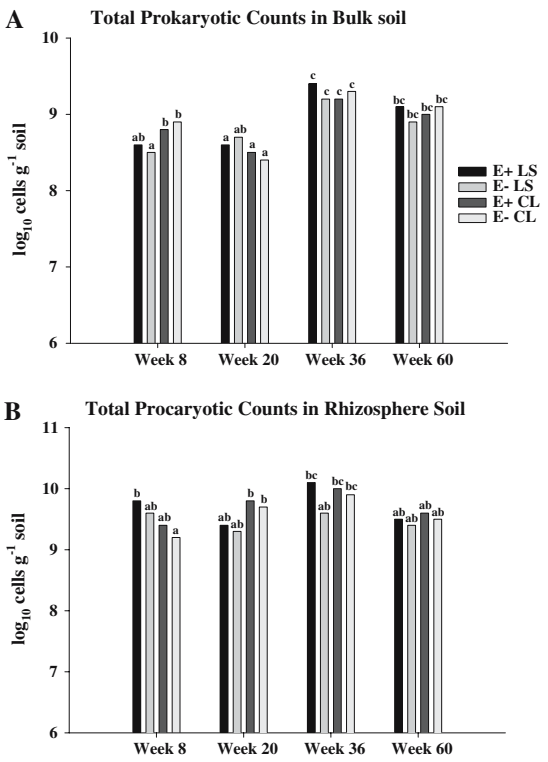

Fig. 1 Total mean prokaryotic cell densities for the four treatments at each sampling week for **A**. bulk soil, and **B**. rhizosphere soil. Different letters above the bars indicate least square differences between treatments at $P < 0.10$

counts in bulk CL (Table 2). Although differences in bulk soil eubacterial cell counts of each treatment were observed between sampling weeks, the pattern did not parallel that of the bulk soil TCs and may have reflected an interaction with soil type (Table 2). The fluctuations of bulk soil eubacterial cell counts among sampling weeks may also have been a reflection of an uneven distribution of metabolically active eubacterial cells.

As expected, the eubacterial cell counts for the rhizosphere soil treatments were greater than the bulk soil eubacterial cell counts ($P < 0.01$). Soil type ($P = 0.07$) had a similar effect on rhizosphere soil eubacterial cell densities as presence and absence of the endophyte ($P = 0.09$) (Table 3). In contrast to the bulk soil eubacterial community, rhizosphere soil eubacterial cell counts paralleled the pattern of bulk soil TC across sampling weeks with increases at sampling weeks 36 and 60 in response to root and above ground inputs of tall fescue (Franzluebbers 2006).

Archaeal cell counts with Probe Arch915

In situ hybridization with the domain specific oligonucleotide probe Arch915 showed that E+ tall fescue decreased the bulk CL archaeal cell

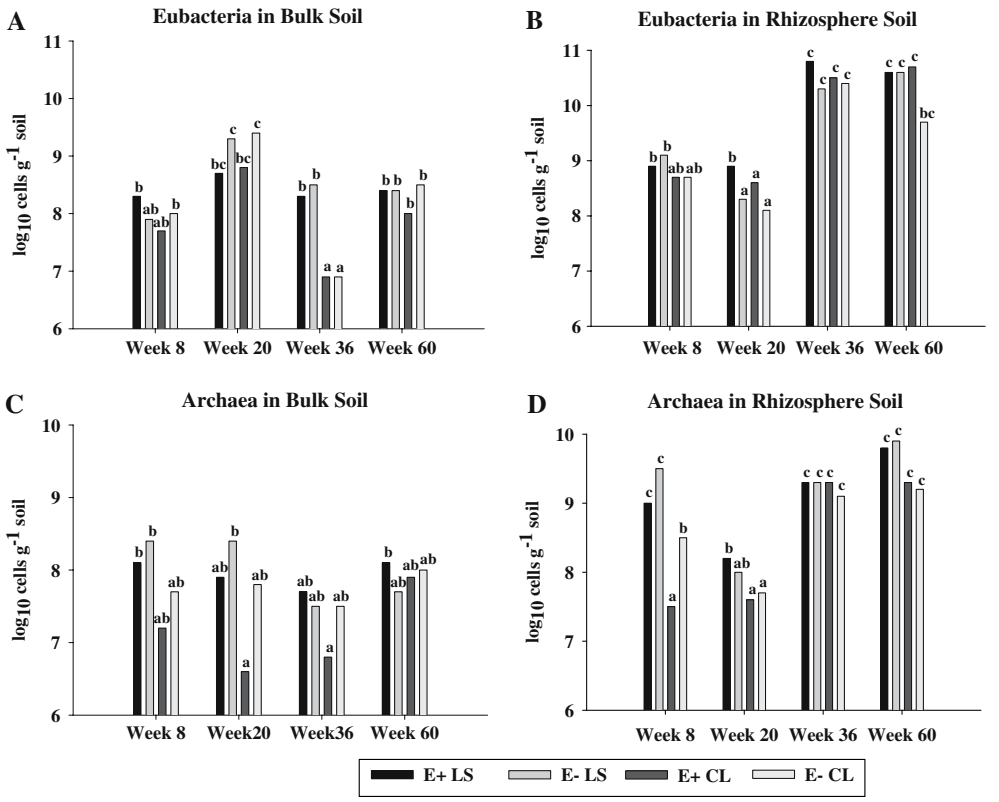

Fig. 2 Mean cell densities for each treatment per sampling week for **A**. Eubacteria in bulk soil, **B**. Eubacteria in rhizosphere soil, **C**. Archaea in bulk soil, and **D**. Archaea in rhizosphere soil. Different letters above the bars indicate least square differences between treatments at $P < 0.10$

count compared to E– tall fescue (Table 2). No difference in bulk archaeal cell count was observed between the endophyte treatments in LS. Differences in archaeal cell counts were observed between the bulk and rhizosphere CL and LS treatments (Tables 2 and 3). The archaeal cell counts from the rhizosphere soils were an order of magnitude greater ($P < 0.01$) than the bulk soil archaeal cell counts and thus displayed a rhizosphere effect. Unlike the bulk soil Archaea (Table 2, Fig. 2C) differences in rhizosphere soil archaeal cell counts were observed between sampling weeks (Table 3). Increases ($P \leq 0.05$) in cell counts for the four treatments occurred between sampling weeks 20 and 36 (Fig. 2D).

In contrast to the eubacterial community, the presence of the endophyte affected the bulk CL archaeal community. Rhizodeposition from E+ tall fescue and its metabolites may have inhibited a component of the bulk CL archaeal community such as the chemolithotrophic ammonia oxidizing Archaea that may comprise as much as 1–5% of the total prokaryotic community as well as be a dominant component of the nitrifying community (Leininger et al. 2006). A connection may exist between (a) the significant soil type/endophyte infection interaction for particulate organic N in which particulate organic N in CL was relatively greater for E+ than E– tall fescue (Franzluebbers 2006), and (b) the decrease in bulk CL archaeal community with E+ compared with E– (Table 2). The hypothetical suppression of ammonia-oxidizing archaea could inhibit mineralization of organic N compounds and subsequent organic C mineralization.

Unlike the bulk soil archaeal community, the rhizosphere archaeal community was not affected by the presence of the endopohyte. Rhizosphere archaeal cell counts were greater for the LS than the CL treatments. The increase in rhizosphere

archaeal cell counts at sampling weeks 36 and 60 compared to two previous sampling weeks suggested a response to the continued root and above ground inputs of the tall fescue whether infected with the endophyte or not. Although Nicol et al. (2005) found no differences in archaeal community structure between bulk and rhizosphere soils the apparent endophyte effect on the bulk CL archaeal community suggests a key difference between bulk and rhizosphere CL communities in our study.

Bacterial cell counts with probes Alf1b, Bet42a, Gam42a, SRB385, HGC69a, CF319a, and PLa5a

In situ hybridization of the bulk and rhizosphere soils with oligonucleotide probes Alf1b, Bet42a, and Gam42a targeting bacteria of the Alpha-, Beta- and Gamma-Proteobacteria, respectively, indicated no effect of E+ tall fescue within either soil type (Tables 2 and 3). Differences in eubacterial communities between bulk LS and CL were mirrored only by bulk soil alpha- and gamma-proteobacterial communities. Bulk LS alpha- and gamma-proteobacterial cell counts were greater than their bulk CL counterparts. Differences in cell counts between sampling weeks were observed only for the bulk soil Gamma-Proteobacteria which increased in density ($P < 0.05$) between sampling weeks 20 and 36 (data not shown).

Rhizosphere soil Alpha-, Beta, and Gamma-Proteobacteria displayed variable responses to both E+ and E– tall fescue between sampling weeks (Table 3). Rhizosphere alpha-proteobacterial cell counts for both soil types increased between sampling weeks 20 and 36 with increases being sustained into sampling week 60 (data not shown). As indicated by the sampling week by treatment interaction effect (Table 3) the rhizosphere E– LS beta-proteobacterial cell count increased ($P = 0.05$) between sampling weeks 36 and 60, while, in contrast, decreases in cell counts occurred between the same sampling weeks for the rhizosphere E+ CL ($P = 0.03$) and E– CL ($P = 0.01$) treatments (Fig. 3A). The rhizosphere soil Gamma-Proteobacteria also displayed various fluctuations in cell counts over sampling weeks as indicated by the sampling week main effect and sampling week by treatment interaction effect (Table 3). The rhizosphere gamma-proteobacterial cell counts increased for the E+ LS ($P = 0.09$) and E– LS ($P < 0.01$) treatments between sampling weeks 20 and 36; in contrast, cell counts decreased for the E+ CL ($P < 0.01$) and E– CL ($P = 0.07$) treatments between sampling weeks 36 and 60 (Fig. 3B). The rhizosphere LS alpha-, beta, and gamma-proteobacterial cell counts were greater than those of the CL cell counts (Table 3). These differences in the rhizosphere soil treatments and the responses of the Beta- and Gamma Proteobacteria to E+ and E– tall fescue may also be indicative of distinct bulk and rhizosphere LS and CL beta- and gamma-proteobacterial communities.

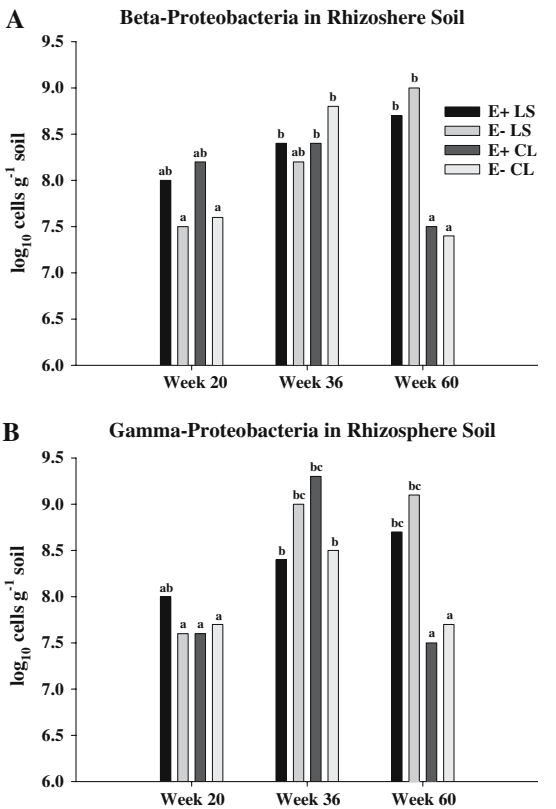

Fig. 3 Mean cell densities for each treatment per sampling week for **A**. Beta-proteobacteria in rhizosphere soil, and **B**. Gamma-proteobacteria in rhizosphere soil. Different letters above the bars indicate least square differences between treatments at $P < 0.10$

Based on preliminary observations that bacterial subdivisions Delta-Proteobacteria, high G + C gram-positive bacteria, Cytophaga-Flavobacteria, and Planctomycetes were near or at the threshold of detection of <6.6 $\log_{10}$ cells g soil^{-1}, analyses were performed for sampling weeks 36 and 60 only. With the exception of Planctomycetes cell counts for E– LS treatment, results from the bulk soil analyses indicated that cell densities for these four components of the soil bacterial community were less than an order of magnitude greater than the threshold of detection (Table 2).

E+ tall fescue did not affect the bulk soil Delta-Proteobacteria, Cytophaga-Flavobacteria, and Planctomycetes communities but did affect the bulk CL high G+C gram-positive bacterial community (Table 2). The attenuation of the bulk CL high G+C gram-positive bacterial community was not mirrored in the response of the bulk CL eubacterial community to E+ tall fescue. Because the high G+C gram-positive bacterial community has a role in the decomposition of complex plant residues (Alexander 1977), its suppression by E+ tall fescue could, therefore, be associated with enhanced C sequestration in bulk soil.

Rhizosphere soil cell counts for these four bacterial subdivisions were an order of magnitude greater ($P < 0.01$) than in bulk soil. The cytophaga-flavobacterial cell count for the rhizosphere E+ LS was greater than the rhizosphere E– LS (Table 3), and may reflect a stimulation of this community by metabolites of the E+ tall fescue as this subdivision of bacteria has been reported to specialize in the degradation of complex macromolecules (Holmes 1991; Reisenbach 1991). The lack of response of the rhizosphere E+ CL cytophaga-flavobacterial community interaction with E+ tall fescue may have been indicative of distinct rhizosphere LS and CL cytophaga-flavobacterial communities.

The sampling week main effect for the rhizosphere soil Delta-Proteobacteria (Table 3) reflected only one difference, a decrease ($P = 0.04$) in cell counts for the E+ CL treatment between sampling weeks 36 and 60 (Fig. 4A). This decline appeared to indicate an inhibitory effect of E+ tall fescue. Although the pooled mean cell counts of the E+ and E– treatments were not different, the increase in cell counts ($P < 0.05$) of the rhizosphere E– LS Planctomycetes community between sampling weeks 36 and 60 contrasted against no change in cell counts of the E+ LS Planctomycetes community (Fig. 4B) may also indicate an inhibitory effect of E+ tall fescue on the rhizosphere LS Planctomycetes community.

Soil type affected the rhizosphere cell counts of the high G+C gram-positive bacteria, Cytophaga-Flavobacteria, and Planctomycetes (Table 3). Like the rhizosphere beta-, and gamma-proteobacterial communities, the rhizosphere high G+C gram-positive bacterial and cytophaga-flavobacterial communities showed various interactions between sampling times and treatments. Only the high G+C gram-positive cell densities of the E– CL treatment increased between sampling weeks

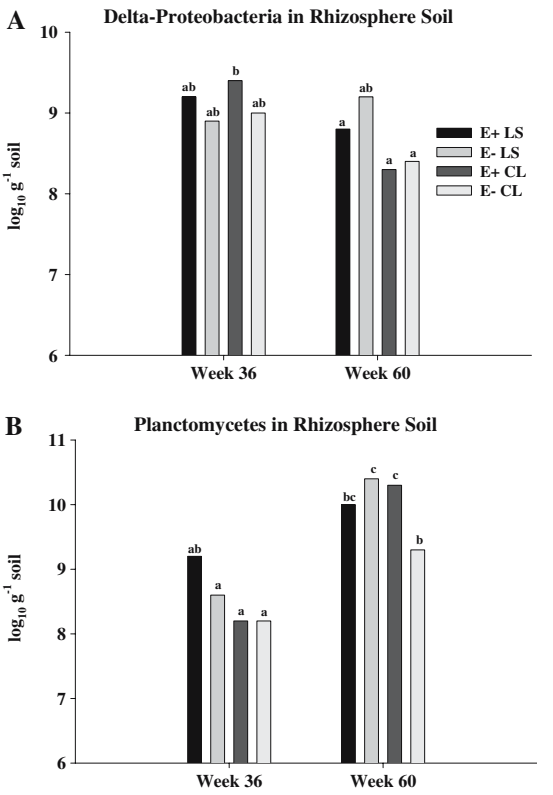

Fig. 4 Mean cell densities for each treatment for sampling weeks 36 and 60 for **A**. Delta-Proteobacteria in rhizosphere soil, and **B**. Planctomycetes in rhizosphere soil. Different letters above the bars indicate least square differences between treatments at $P < 0.10$

36 and 60. The cytophaga-flavobacterial cell counts increased in LS but not CL treatments further indicating differences between cytophaga-flavobacterial communities. The differential responses of the bulk and rhizosphere LS and CL high G+C gram-positive bacterial communities to E+ tall fescue may reflect the environmental responses of distinct bulk and rhizosphere soil high G+C gram-positive bacterial communities, as has been observed in other soil plant systems (Basil et al. 2004; de Ridder-Duine et al. 2005).

General microbiological observations

The percentage of bulk soil eubacterial cells that were enumerated with probe Eub338 ranged from 10% to 40% of the bulk soil TC, and was in the range previously observed in bulk soils (Hahn et al. 1992; Zarda et al. 1997; Chatzinotas et al. 1998; Sandaa et al. 1999). In contrast, the percentage of rhizosphere soil eubacterial cells enumerated ranged from 40% to >100% of the rhizosphere soil TC. Several reasons to account for the observed disparity between cell counts with oligonucleotide probes and total direct counts are possible. Probe Eub338 may not have hybridized with all Eubacteria (Zarda et al. 1997; Neef et al. 1998; Daims et al. 2001; Sandaa et al. 1999); some cells may have been impermeable to oligonucleotide probes (Zarda et al. 1997); the target site on the rRNA molecule may have been inaccessible (Amann 1995); and low cellular rRNA content could have generated a non-detectable signal (Zarda et al. 1997). The increase in rhizosphere soil eubacterial percentage of TC was most likely the result of rhizosphere stimulation and may also have indicated the stimulation of less abundant members of the eubacterial community not otherwise detected in the bulk soil (Gans et al. 2005). DAPI stained cells outnumbered the cells targeted by the fluorescent oligonucleotide probes. Cells stained with fluorescent oligonucleotide probes were generally brighter than the same cells stained with DAPI (Alfreider et al. 1996). The dimmer DAPI signals may be attributable to the absorption of DAPI emission by Cy3 (Llobet-Brossa et al. 1998). Although cells detected with in situ hybridization were metabolically active, TC for both bulk and rhizosphere soils could have been underestimated. Underestimations could be attributable to low DAPI signals because of the size and physiological state of the microbial community (Roszak and Colwell 1987), and in some cases the faintness of DAPI fluorescence may have been indistinguishable from background autofluorescence.

The percentage of bulk soil archaeal cells enumerated with probe Arch915 ranged from 2% to 13% of bulk soil TC. This range was greater than the 2% of TC previously observed in bulk soils (Zarda et al. 1997; Chatzinotas et al. 1998; Sandaa et al. 1999). Rhizosphere soil archaeal cells ranged from 5% to 50% of the rhizosphere soil TC. Greater rhizosphere soil archaeal cell percentage of TC indicated a proportional increase in archaeal cells over eubacterial cells in response to rhizosphere activity. Our observations of Archaea substantiate reports that Archaea are components of the soil and rhizosphere microbial communities (Zarda et al. 1997; Sandaa et al. 1999; Simon et al. 2000; Sliwinski and Goodman 2004; Leininger et al. 2006), and that they are ubiquitous in terrestrial habitats. Our observations indicated that the archaeal community can be affected by both soil type and plant cultivar.

Most of the cells visualized with the domain oligonucleotide probes were rod-shaped of various dimensions (data not shown). Some were curved as exemplified by the beta-proteobacterial microcolony (Fig. 5A); some cells displayed various rod-like shapes as exemplified by the Alpha-Proteobacteria (Fig. 5C). Little or no morphological diversity was observed among the Planctomycetes between two different rhizosphere soil samples (Fig. 6A–D). Some rod-shaped bacteria were observed with probe Pla5a, but these were not enumerated as we assumed they were the result of weak cross-reactivity and since no rod-shaped members of this group have ever been observed. Cells in microscopic fields were either in clumps or microcolonies as observed in Fig. 5A and B, or dispersed as seen in Fig. 5C and D.

No dominant subdivisions of bacteria appeared in the bulk soil samples over the sampling weeks, which differed from the observations of Zarda et al.

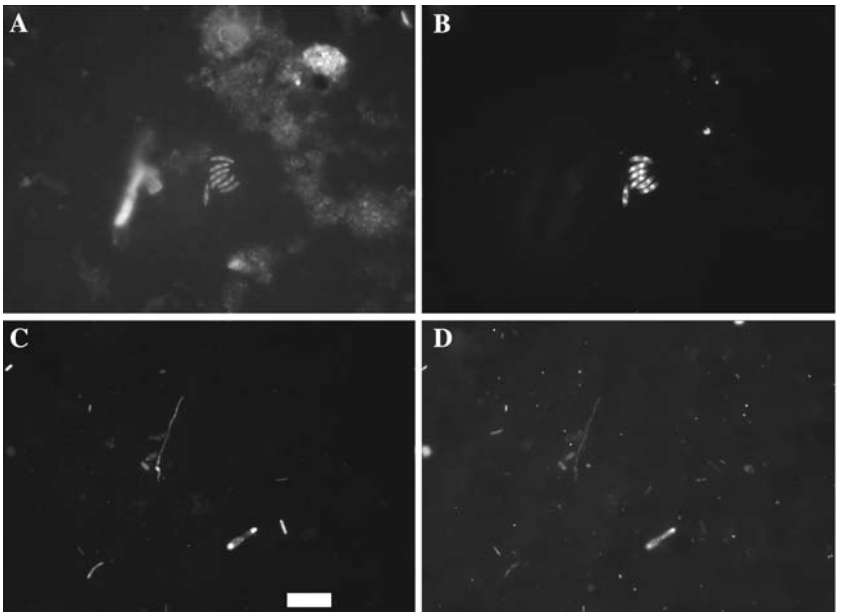

Fig. 5 Epifluorescent micrographs of bacteria from rhizosphere soil samples at sampling week 60 detected by probe Bet42a (**A**) and its DAPI stained counterpart (**B**), and by probe Alf1b (**C**) and its DAPI stained counterpart (**D**). Bar equals 6 μm

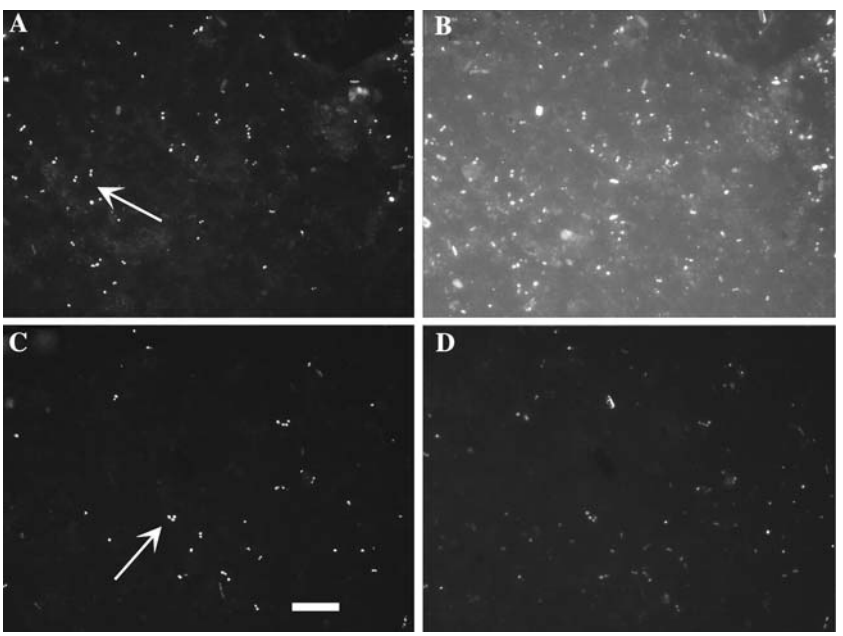

Fig. 6 Epifluorescent micrographs of bacteria from two rhizosphere soil samples at sampling week 60 detected by probe Pla5a (**A** and **C**) and their DAPI stained counterparts (**B** and **D**). Arrows point to typical Planctomycetes cells. Bar equals 6 μm

(1997) who reported substantially greater cell counts for the alpha- and delta-proteobacteria and Planctomycetes. Cell counts of Planctomycetes for the rhizosphere soil samples, however, appeared to be a greater percentage of the bacterial community than other bacterial subdivisions. This observation further supports those of other researchers that the Planctomycetes community appears to be a substantial component of the soil microbial community (Liesback and Stackebrandt 1992; Stackebrandt et al. 1993; Borneman et al. 1996; Lee et al. 1996; Zarda et al. 1997).

Conclusions

Endophyte-infected tall fescue appeared to suppress the archaea and high G+C gram-positive

communities in the bulk clay loam, decreased the rhizosphere cell counts of the delta-proteobacterial community between sampling weeks 36 and 60 in the clay loam, and appeared to inhibit the rhizosphere Planctomycetes community in the loamy sand compared to the endophyte-free tall fescue. Any inhibitory effect of E+ tall fescue on these components of the soil prokaryotic community had a minimal effect on the rapid accumulation of soil C over the relatively short duration of this mesocosm study (Franzluebbers 2006). In the long-term, however, E+ tall fescue's apparent suppression of these soil microbial components may be a factor in its enhancement of soil carbon sequestration. Microbial response to the E+ as apposed to the E– tall fescue was affected by soil type. This observation was not surprising considering that the LS and CL soils were distinct in physical characteristics and geographic location, that each soil type most likely, therefore, contained microbial components of different metacommunities (Curtis and Sloan 2004), and that other rhizosphere bacterial communities have been shown to be distinct from their bulk soil community (Alexander 1977; Basil et al. 2004; de Ridder-Duine et al. 2005).

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